

Chapter 17

Synthesis of Beeswax

Abstract The notion that honeybees secrete wax and not gather it from blossoms was first shown in the mid-18th century (Hornbostel 1744). Later, Huber (1814) observed that newly settled swarms do not gather pollen but construct combs, and he concluded that beeswax was the secretory product of the glands of the wax mirrors and fuelled by honey. However, the actual amount of fatty material present in bees, before and after their incarceration in experimental cages and in combs constructed in the interim, had to be determined. This Dumas and Edwards (1843) did, and they concluded that the amount of fatty material present at the onset of the experiment could not account for the wax produced by the end of the experiment; hence bees both synthesise and secrete wax. A century later, Piek (1961, 1964) fed captive bees (1-¹⁴C)-acetate, (UL-¹⁴C)-glucose and deuterated water and recovered the labels both from bees and newly constructed combs. Lambremont and Wykle (1979) incubated homogenates of the wax glands with (1-³H)-tetracosanol and recovered the label only in the wax ester fraction and the ³H wax ester fraction, which yielded a ³H-fatty alcohol with the same R_f value as authentic tetracosanol. Blomquist and Ries (1979) showed that long-chain primary alcohols, fatty acids and the acyl group of acyl-CoA were incorporated in wax monoesters, and that (1-¹⁴C)-palmitate entry into the monoester pool was enhanced by ATP, CoA and MgCl₂, while the addition of palmitoyl-CoA resulted in a fivefold yield increase. Subsequently, the specific cellular sites for the origin of hydrocarbons and fatty acids within the wax gland complex and the necessary ultrastructural correlates of this activity and of their transport, were determined Hepburn et al. (1991).

17.1 Introduction: Proof of Beeswax Synthesis

At the outset of this chapter, it must be pointed out that all published work on the synthesis of beeswax by honeybees has been restricted to *A. mellifera*; none of the Asian species have been examined in this regard as yet. More than two centuries



Fig. 17.1 François Huber (2 July 1750–22 December 1831) was a blind Swiss naturalist who, with the assistance of François Burnens, was able to carry out investigations that laid the foundations of scientific knowledge of the life history of the honeybee. His *Nouvelles Observations sur les abeilles* was published in Geneva in 1792, and was revised and published in 1814 (this was the edition that was used by CP Dadant for an English translation in 1926). It is in this volume that Huber described, in considerable detail, the construction of comb and experiments on the respiration of bees. It remains a modern honeybee text and is still cited today

ago, careful observations and shrewd inferences led Hornbostel (1744), and then Hunter (1792), to conclude that honeybees secrete wax, a view that ran counter to the 2000 year-old belief that bees gather wax from blossoms, as was promulgated by Aristotle (Fraser 1931). Having reached the same conclusion, quite independently, Huber (1814) (Fig. 17.1) attempted an experiment to show that bees actually synthesise wax rather than merely secrete it as a chyme-treated, transmuted form of pollen. Added to this was the contentious problem of the route of secretion: was wax secreted from the proboscis (de Réaumur 1740), the anus (Dobbs 1750), or from the wax mirrors, as Hornbostel, Hunter and Huber believed?

17.1.1 François Huber (1814)

A key piece of evidence that led to Huber's experiments were observations, by both de Réaumur and Hunter, that newly settled swarms do not gather pollen, but avidly construct combs, whereas old established colonies readily gather pollen.

Huber converted these observations into a series of experiments. He fully appreciated that the time element must be such as to preclude the elaboration of wax from pollen that might have been ingested before the experiment. Huber hived a swarm in a wax-free skep, and placed it in a room where the bees were given water and honey, but no pollen. Five days later, the bees had eaten the honey and produced new combs. He repeated this experiment for about a month, and always obtained the same result: a continuous supply of honey was sufficient for comb construction to proceed. He then mounted the reciprocal experiment and fed pollen to the bees but not honey. After 8 days he found neither any combs in the skeps nor wax scales on any of the bees. He concluded correctly, if prematurely (cf. below), that beeswax was the secretory product of the glands of the wax mirrors and that the fuel for synthesis was honey, wax was not made from pollen.

Huber anticipated any objections that may arise (the honey he fed his bees might be contaminated with wax), and performed a complementary experiment. Oddly enough he did not mention pollen contamination of honey. He incarcerated three colonies and fed one of them syrup made from white sugar, another syrup from brown sugar, and the third honey. Eight replicated feeding trials always resulted in the production of wax combs in the apparent absence of pollen. Unfortunately, in attempting to discredit pollen as the source of wax, Huber missed the significance of pollen as an essential source of protein. His experiments certainly showed that sugar drives wax secretion, even if his conclusions about pollen were equivocal. Berzelius and Thénard, distinguished academic chemists of the day, regarded Huber's conclusions with reserve, and rightly stated that it had not been conclusively shown that bees have the faculty to produce wax (Holmes 1985); nor had any other animal been shown to be capable of synthesising lipids (Dumas and Edwards 1843).

17.1.2 The Chemists: Dumas and Edwards (1843)

To legitimise the claims of Huber, it was clearly necessary to determine the actual amount of fatty material present in bees before their incarceration in any experimental cage and again at the end of the experiment, as well as that contained in any combs which may have been constructed in the interim. After a few false starts, Dumas and Edwards (1843) hived a small swarm of bees, having removed 5 % to sample for fat analysis. They determined the mean fat content and mass of the standing population of the colony, and of samples obtained from three other such colonies. They proceeded to measure: (1) the wax content of the honey which they fed to their other colonies of confined bees; (2) collected wax scales dropped on the floor of the hive; (3) the amount of comb produced over the 11 days of the experiment; (4) the amount of fatty material contained in larvae and eggs present in the comb, and finally; (5) they measured the amount of fatty material present in the bodies of the bees at the end of the experiment. The results from the experiment are shown in Table 17.1.

Table 17.1 Results obtained by Dumas and Edwards (1843) in their experiment to prove that honeybee workers can synthesise wax

	Input (mg)	Output (mg)
1. Mean fatty material per bee at the beginning of the experiment	1.80	
2. Wax particles in honey fed to bees did not exceed	0.38	
3. Mean quantity of fatty substance available per bee (1 + 2)	2.20	
4. Mass of wax produced per bee during the experiment (2 + 3)		6.40
5. Mean fat content of bees at the end of the experiment (5)		4.30
6. Balance (4 + 5 - 3)		8.50

They concluded that the amount of fatty material present at the onset of the experiment, both as body fat and wax present in honey, was insufficient to account for the amount of wax produced by the end of the experiment; hence bees both synthesise and secrete wax.

17.2 Routes of Synthesis

Of far greater importance than the proof that honeybees synthesise wax, was the demonstration for the first time by Dumas and Edwards (1843) that *an animal* could synthesise fats. This was a burning issue among chemists of the 1840 period, championed on theoretical grounds by the German chemist, Justus von Liebig (Fig. 17.2), and actually opposed by the French under the leadership of Jean-Baptiste Dumas (Fig. 17.3). Thus it is only fitting that Dumas and Edwards should have answered this question in their ‘balance sheet’ studies of bees. Oddly enough, Dumas and Edwards did not consider their results from bees to hold any significance to the questions as to whether animal are able to synthesise fats. They, and other academicians of their day, were only willing to accord this trait to animals after it had been shown to be so shortly after with Persoz’s experiments with geese, and Boussingault’s work on pigs (Florkin 1977; McCosh 1984). The confirmation by Dumas and Edwards that sugar or honey was sufficient for the secretion of wax and building of comb, gradually seeped into the general apicultural literature as a method for producing wax (Langstroth 1853; Dzierzon 1861), and soon after, the first attempts to quantify the energetics of the process appeared.

17.3 Biochemical Investigations on Beeswax Synthesis

17.3.1 Hypothetical Scheme for Beeswax Synthesis

A thorough analysis of the synthesis of beeswax is clearly predicated on knowledge of its composition; and, while studies on the latter had been in progress for a good 150 years (Grün and Halden 1929), real headway was only made

Fig. 17.2 Justus Freiherr von Liebig (12 May 1803–18 April 1873) was a German chemist who made major contributions to agricultural and biological chemistry. On theoretical grounds he championed the idea that animals could synthesize lipids, and was proven right with the works of Dumas and Edwards (1843) on honeybees, Persoz (1843) on geese, and Boussingault (1843) on pigs, as cited by McCosh (1984)

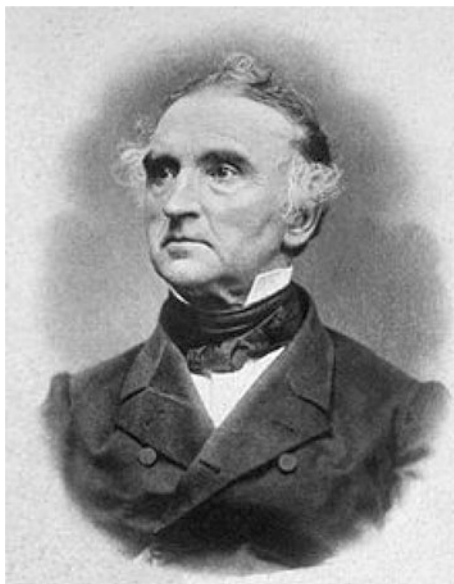


Fig. 17.3 Jean Baptiste André Dumas (14 July 1800–10 April 1884) was a French chemist, renowned for his experiments on organic analysis and synthesis and the determination of relative atomic masses and molecular weights. Although opposed to the idea that animals might synthesize lipids on theoretical grounds, his experiments with honeybees, published in 1843, provided the first proof that an animal could synthesize lipids



intermittently over the past 75 years. Following an extensive series of wax analyses, Chibnall and Piper (1934) and Chibnall et al. (1934) postulated that the primary alcohols of beeswax are formed as reduction products of the corresponding acids. They also suggested that the hydrocarbons arise through decarboxylation of the corresponding acids, hypotheses that remained untested. In an early series of experiments, Piek (1961, 1964) investigated wax synthesis in or

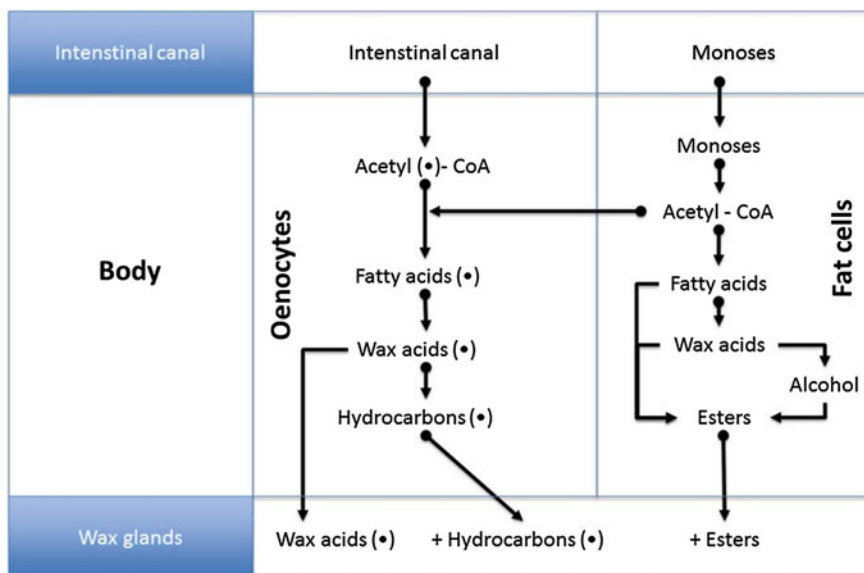


Fig. 17.4 Hypothetical scheme for the synthesis of beeswax based on feeding *A. mellifera* bees with labelled acetate. Asterisks indicate the fate of labelled ^{14}C -labelled material (Piek 1964)

which he fed captive bees ($1\text{-}^{14}\text{C}$)-acetate, ($\text{UL-}^{14}\text{C}$)-glucose and deuterated water for two weeks, and then recovered the labels from both the bees and the newly constructed combs.

Piek found that both the hydrocarbon and free acid fractions were labelled, but was unable to measure any activity in either the fatty alcohols or wax esters. Coupling his results with the prevailing histological picture of the wax gland complex and the views of Chibnall and Piper (1934), Piek proposed a hypothetical scheme for the synthesis of wax. He concluded that esters are produced by the fat cells (they did not take up acetate, but sequestered monoses from the haemolymph), the hydrocarbons and free wax acids by the oenocytes; the products of both tissues being delivered to the wax glands on the surface of the cuticle as shown schematically in Fig. 17.4.

That Piek did not recover any labelled material in either the ester or alcohol fractions is, in retrospect, possibly attributable to the low specific activity of his starting material and/or to the loss of label through other metabolic pathways. Young (1963) had touched on this problem by injecting ($2\text{-}^{14}\text{C}$)-acetate into the body cavities of bees rather than feeding them. Twelve hours after injection he recovered labelled material in both the wax ester and free acid fractions of beeswax scales. Young's results are obviously inconsistent with Piek's conclusion that the fat body cannot metabolise acetate; but, the possibility of partitioning wax synthesis in different tissues prior to secretion, remained an untested and viable possibility.

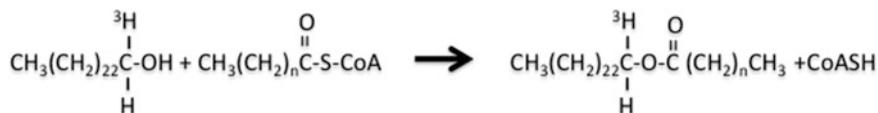


Fig. 17.5 Proposed route for monoester synthesis in *A. mellifera* (Lambreton and Wykle 1979)

17.3.2 Monoester Synthesis

Two refinements in the study of wax synthesis appeared somewhat later, when Lambremont and Wykle (1979) prepared homogenates of worker bees' wax glands and incubated their cell-free preparations with (1-³H)-tetracosanol. The metabolism of this primary alcohol is such that the label is only known to be recoverable as tritiated water, and the unmetabolised alcohol in wax esters derived from the alcohol. After incubation, they recovered the label only in the wax ester fraction, and showed that the ³H wax ester fraction yielded a ³H-fatty alcohol having the same chromatographic R_f value as authentic tetracosanol. The labelled ester also had the same chromatographic mobility as those of other wax monoesters, which had previously been shown by Tulloch (1970) to consist mainly of palmitates of C₂₄–C₃₄ alcohols.

Lambreton and Wykle (1979) concluded that their labelled ester was most probably the monoester, tetracosyl hexadecanoate. They established that the enzyme activity of their preparation was functional between a pH of 6.5–7.4, with maximum activity at a pH of 7.1 at 37 °C, and that Coenzyme A and Mg²⁺ are cofactors in a monoester synthesis driven by ATP. The temperature value of 37 °C for maximum enzyme activity is within 5 % of brood nest temperature and of wax producing bees (Hepburn and Muller 1988). Lambremont and Wykle regarded the enzymes that synthesise the wax esters as not specific for long chain alcohols, because both hexadecanol and tetracosanol were readily incorporated in ester synthesis. Lambremont and Wykle (1979) claimed that tetracosanol and short-chain alcohols are absent from wax monoesters, possibly because the wax gland does not form shorter chain alcohols as opposed to the specificity characteristics of the relevant enzymes. This is open to debate in view of Tulloch's report (1971) on the presence of tetracosanol in commercial samples of beeswax. Finally, Lambremont and Wykle (1979) suggested a route for monoester synthesis that had been previously shown to occur in other animals and in plants (Fig. 17.5).

In a parallel study of ester synthesis, Blomquist and Ries (1979) also used a microsomal preparation of workers' wax glands to investigate the incorporation of long-chain primary alcohols, fatty acids and the acyl group of acyl-CoA into wax monoesters. They showed that (1-¹⁴C)-palmitate entry into the monoester pool was enhanced by ATP, CoA and MgCl₂, while the addition of palmitoyl-CoA resulted in a fivefold increase in monoester synthesis when labelled tetracosanol was used as the substrate. Accordingly, they concluded that the acyl group of acyl-CoA is transferred to the primary alcohol during the synthesis of monoesters. The works of

both Lambremont and Wykle (1979) and Blomquist and Ries (1979) were based entirely on the recovery of reaction products from microsomal preparations, and the methods and the conclusions they reached were the same. However, neither study had precluded the possible synthesis of epicuticular lipids as distinct from those of wax scales, until Blomquist et al. (1980) specifically addressed this problem.

17.3.3 *Cuticular and Comb Waxes*

The composition of the wax of the epicuticle of worker honeybees differs quantitatively from that of comb wax (Lockey 1991). While the major component of the epicuticular lipids is hydrocarbon (~58 %), the hydrocarbon content of comb wax is relatively low (~13–17 %) as monoesters comprise the largest component (Tulloch 1971). Blomquist et al. (1980) analysed the major fractions of the epicuticular waxes by gas–liquid chromatography, and found that they were qualitatively similar to those of comb wax. When Blomquist et al. (1980) injected radio-labeled acetate into worker honeybees that were not actively producing comb, they recovered much of the radioactivity in the hydrocarbon fraction. In bees actively producing comb wax, a higher percentage of radioactive products were recovered in the monoester fraction.

Blomquist et al. (1980) also recorded the dramatic effect of age on the distribution of radioactivity from acetate into the various wax fractions from honeybees studied during the summer months. The major wax component synthesized by the wax-secreting age group was monoester, while in both younger and older bees hydrocarbon was the major wax component formed (Fig. 17.6). Both in vivo and in vitro experiments, using insects actively producing comb wax, showed that the abdomen produced significant amounts of monoester, hydrocarbon and other esters, whereas the thorax synthesized mostly hydrocarbon. These data show that the epidermal cells and wax glands each produce a wax with a distinct composition, and that the age and seasonal differences observed in wax synthesis are due to the presence or absence of active wax glands (Blomquist et al. 1980).

Using winter bees, which are not actively engaged in any significant comb-building (although some wax scales are still secreted—Cassier and Lensky 1995), Blomquist et al. (1980) demonstrated that the cuticular waxes, while qualitatively similar in composition to comb wax, differ quantitatively as shown in Table 17.2.

Comb wax is considerably poorer in hydrocarbons but moderately richer in monoesters than the epicuticular waxes of worker honeybees. Turning to the synthesis of wax, Blomquist et al. (1980) proceeded to show that more label could be recovered from the hydrocarbon fraction of bees not actively secreting wax and, conversely, that more label could be recovered from the monoester products of bees actively secreting wax (Table 17.3). Similarly, an analysis of summer bees, (workers raised during spring and the beginning of summer), showed that the greatest amount of labelled acetate was recovered from 11- to 18-day-old bees, those at the peak of wax production (Rösch 1927; King 1928), while labelled

Fig. 17.6 Distribution of ($1-^{14}\text{C}$)-acetate in the hydrocarbon and monoester fractions of *A. mellifera* worker bees of wax secreting age. *H* hydrocarbon, *M* monoester (Blomquist et al. 1980)

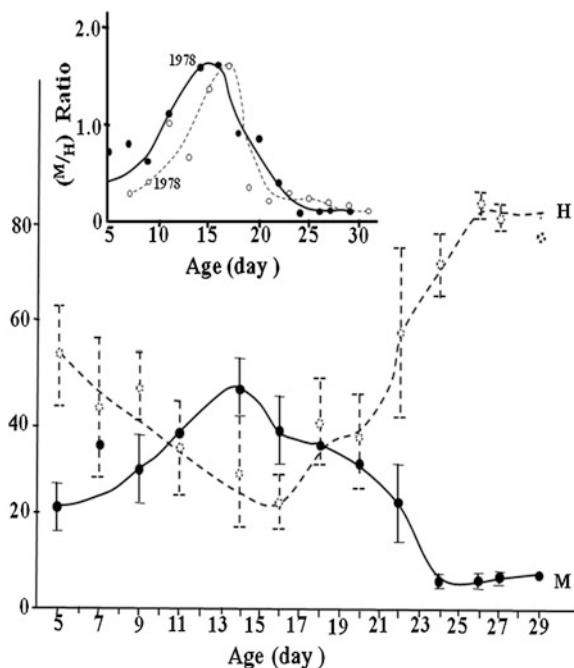


Table 17.2 Chemical composition of *A. mellifera* cuticular and comb waxes (Blomquist et al. 1980)

Class	Percent by weight	
	Cuticular wax	Comb wax
Hydrocarbon	58	13–17
Monoester	23	31–35
Diester	9	10–14
Triester	2	
Free fatty acids		3
More polar material	9	34

hydrocarbons dominated the products of both younger and older bees. Against this, they also found that the relative composition of the hydrocarbon pool varied with age (Table 17.4), and independently of whether the bees were actively secreting wax. The hydrocarbon pool also varied seasonally. Finally, on the evidence that the hydrocarbons are derived from acetate, Blomquist et al. (1980) suggested that the (*Z*)- C_{23} – C_{29} alkenes are derived from fatty acids desaturated at the 9 position, while those desaturated at the 8 and 10 positions serve as intermediates in the formation of longer chain alkenes.

A most interesting piece of natural history traced by Blomquist et al. (1980) involved monitoring the distribution of 1-($1-^{14}\text{C}$)-acetate in both the monoester and hydrocarbon fractions of adult worker bees (Fig. 17.7). They noted that the rise and fall in wax secretion is closely related to that of labelled monoester

Table 17.3 Incorporation of (1-¹⁴C)-acetate into *A. mellifera* comb wax (Blomquist et al. 1980)

Class	Percent distribution	
	Insects not producing Comb wax ^a	Insects actively producing Comb wax ^b
Hydrocarbon	47 ± 3 ^c	32 ± 6
Monoester	18 ± 2	35 ± 7
Diester	7 ± 1	9 ± 2
Triester	12 ± 1	3 ± 1
Free fatty acids		
More polar lipid	16 ± 3	21 ± 4

^a Insects used in April 1978 when they were not actively producing comb wax

^b Insects were actively producing comb wax as evidenced by wax scales on the ventral abdomen. Insects were used in June 1978

^c Values are the mean ± S.D. Five groups of five insects per group were used in each experiment

Table 17.4 Changes in the composition of the major hydrocarbons in *A. mellifera* with worker age (Blomquist et al. 1980)

Hydrocarbon component	Percentage composition					
	7 days	9 days	16 days	18 days	22 days	26 days
nC ₂₃	2.8 ^a	2.5	2.3	4.1	3.8	25.8
nC _{23:1}	0.7	0.4	0.3	0.2	1.1	4.6
nC ₂₅	3.0	3.0	2.5	5.7	5.7	27.2
nC _{25:1}	1.3	0.9	1.1	1.3	2.3	7.6
nC ₂₇	7.8	9.4	7.8	11.9	14.5	12.4
nC _{27:1}	0.7	0.3	0.2	3.2	1.1	1.8
nC ₂₉	7.1	10.4	9.9	10.0	9.0	6.8
nC _{29:1}	1.8	1.6	2.2	1.1	2.6	0.8
nC ₃₁	6.9	8.5	8.3	7.5	5.7	3.6
nC _{31:1}	19.4	17.7	22.8	19.6	17.2	3.2
nC ₃₃	1.4	1.5	1.4	1.0	1.0	0.4
nC _{33:1}	43.6	41.2	40.1	32.4	35.2	5.6
nC _{35:1}	3.5	2.6	1.1	0.7	0.8	0.2
% Saturated	29.0	35.3	32.2	40.2	39.7	76.2
% Unsaturated	71.0	64.7	67.8	59.8	60.3	23.8
% C ₂₃ –C ₂₉ Components	25.2	28.5	26.3	37.5	40.1	87.0
% C ₃₁ –C ₃₅ Components	74.8	71.5	73.7	62.5	59.9	13.0

^a Values are the mean of two groups of fifteen insects per group. The range for each value was less than 15 % of the mean

synthesis, and, conversely, to that of hydrocarbon synthesis. This observation is in accordance with the fact that the increase in monoester synthesis, observed in bees during the northern summer which are actively producing wax, is absent from autumnal bees, which normally do not produce wax, nor show any increase in monoester synthesis. These few and hard-won battles towards unravelling wax synthesis in honeybees might well gain impetus from the striking advances made

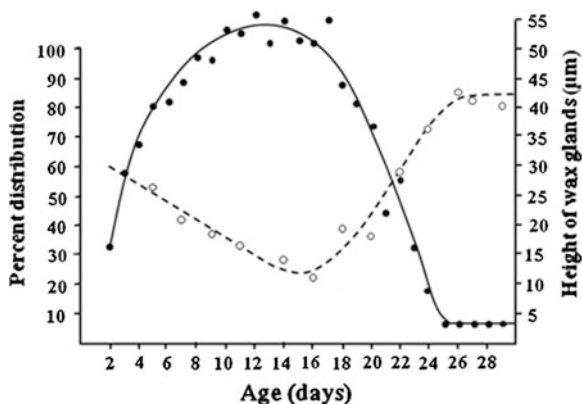


Fig. 17.7 Distribution of (1-¹⁴C)-acetate in the hydrocarbon fraction (*open circles*) of *A. mellifera* worker honeybees and the corresponding rise and fall of the epithelium (*closed circles*) of the wax gland in relation to worker age (Hydrocarbon data from Blomquist et al. 1980; wax gland data from Rösch 1927)

by plant chemists in recent years. That many of the components of wax are synthesised from acetate is now accepted as a general principle.

The older view of Chibnall et al. (1934) has been supplanted by the discovery by Kolattukudy (1967a): hydrocarbons and their derivatives are produced by an 'elongation-decarboxylation' mechanism, by which fatty acid synthetase (a multi-enzyme protein that catalyzes fatty acid synthesis) produces palmitic acid. This end-product is elongated through the addition of C₂ units until the growing chain is eventually decarboxylated with the release of hydrocarbons, a pathway which precludes both ketones and secondary alcohols from being sources of wax hydrocarbons. Kolattukudy (1968) further suggested that there are chain-elongating enzymes with different specificities, but this idea awaits confirmation. The origin of fatty alcohols from exogenous fatty acids has also been confirmed with the discovery of fatty acyl-CoA reductase (Kolattukudy 1969). Finally, Kolattukudy (1967b) demonstrated the existence of a protein which catalyses an acyl-CoA-dependent esterification of fatty alcohols giving rise to wax esters. The biosynthesis of long-chain fatty acids requires fatty acid synthetase; however, exogenous units ranging in length from C₂ to C₂₄ can be incorporated in syntheses by plants, for which malonyl-CoA is the elongating agent and NADPH the reductant (Kolattukudy et al. 1976) (Fig. 17.8).

17.4 Cellular Basis of Synthesis

Since the work of Clements (1959) on the fat body of locusts, the adipocytes have emerged as the major seat of intermediary metabolism in insects (Candy 1985; Keeley 1985). The peripheral adipocytes, such as those associated with the wax

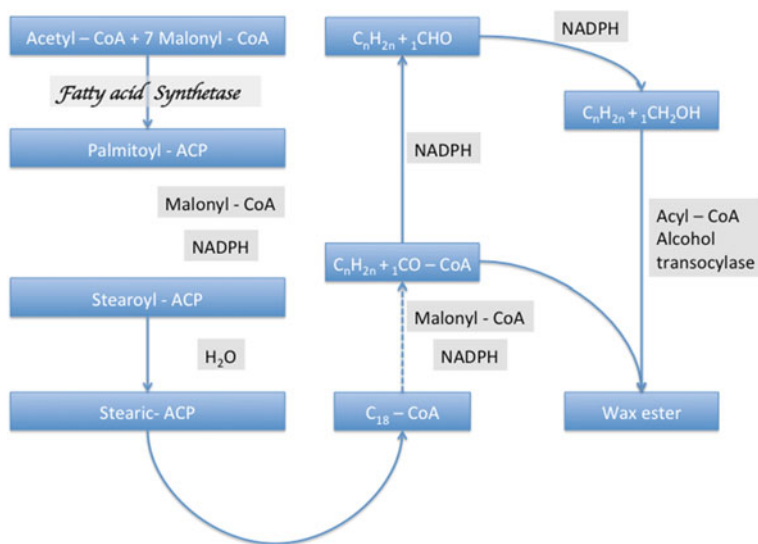


Fig. 17.8 Proposed route for the synthesis of wax esters in plants (Kolattukudy 1980)

mirror epidermis, are regarded as the primary site of lipid synthesis and storage (Dean et al. 1985). However, production of specific classes of compounds is not understood. In studies of hydrocarbon synthesis, Diehl (1973, 1975) demonstrated that the oenocytes of locusts produce hydrocarbons from acetate. Chino (1985) showed that lipophorins transport both hydrocarbons and diacylglycerol from the oenocytes to the cuticle in cockroaches. Similarly, the elongation-decarboxylation of long chain fatty acids, proposed by Kolattukudy (1967a), has found support in the synthesis of alkanes (Major and Blomquist 1978) and alkenes (Dwyer et al. 1981) in cockroaches.

Because cellular synthesis ultimately depends on mitochondrial respiration, the possibility of neuroendocrine control in relation to wax synthesis must be briefly considered. Altmann (1959) showed that extracts of the corpora allata from laying workers increased the respiratory rate of normal queenright workers, a result clouded by the stimulation of ovarian activation in the recipients. This problem was ultimately clarified in other insects by Slama (1964) and Wiens and Gilbert (1965), who showed that respiration at the cellular level is controlled by the corpora cardiaca. There is also evidence that lipogenesis in the insect fat body is stimulated by the corpora cardiaca (Downer and Steele 1972), and possibly governed by juvenile hormone, since allatectomy results in high levels of lipid production (Gilbert 1967; Steele 1985).

While lipogenesis proceeds from carbohydrate precursors in the fat body (Chino and Gilbert 1965), the rate-limiting reaction in the conversion of glucose to lipid has not been identified. Still, extracts of the corpora allata accelerate glycolysis (Steele 1985). It is also of interest that 20-hydroxyecdysone stimulates

Table 17.5 Changes in hydrocarbons in the wax gland epidermis of the Cape honeybee, *A. m. capensis*, workers with age (Hepburn et al. 1991)

Hydrocarbon	Bee age (days)							
	0	3	6	9	12	15	18	21
25:0	20.6	22.3	21.9	21.3	21.6	24.0	23.2	22.5
27:0	39.7	37.0	33.3	35.0	33.6	37.4	37.8	43.7
29:0	6.9	7.4	8.3	9.7	8.4	10.4	12.2	9.9
31:0	4.4	2.5	3.1	3.9	3.7	2.1	3.7	4.2
33:0	10.3	11.1	12.5	14.6	15.9	12.5	13.4	12.7
35:0	2.9	3.7	2.1	1.9	3.7	3.1	2.4	2.8
Total	83.8	84.0	81.2	86.4	86.9	89.5	92.7	95.8
33:1	4.4	3.7	6.3	5.8	5.6	4.2	2.4	1.4
35:1	11.8	12.3	12.5	7.8	7.5	6.3	4.9	2.8
Total	16.2	16.0	18.8	3.6	13.1	10.5	7.3	4.2

hydrocarbon synthesis in flies (Arnold and Regnier 1975), and that the oenocytes of a beetle can synthesise ecdysteroids (Romer et al. 1974). Finally, Gast (1967), and then Robinson (1985), showed that the implantation of active corpora allata into young bees resulted in the hypotrophy of hypopharyngeal glands and the movement of bees away from brood care, while allatectomy extended the life of these glands (Imboden and Luscher 1975). However, somewhat later, Muller and Hepburn (1994) experimentally established, by allatectomy and corpora allata implants, that neither Juvenile Hormone III nor the corpora allata play a role in regulating either the onset of wax secretion nor the amount of wax secreted.

17.4.1 Chemical Composition and the Ages of Worker Bees

Hepburn et al. (1991) conducted studies on wax synthesis and secretion in honeybees to identify specific cellular sites for the origin of hydrocarbons and fatty acids within the wax gland complex, and to establish the necessary ultrastructural correlates of this activity and of their transport. Of equal importance, they measured the actual rate of wax secretion in bees of different ages, to assess how well chemical composition of the tissues and cycles of ultrastructural change corresponded with the cycles of wax production within the division of labour. They developed a technique to isolate the epidermis, oenocytes and adipocytes, and were able to study each tissue separately. The hydrocarbons and fatty acids of the epidermis and oenocytes were analyzed in bees of the same age as those used in the ultrastructural studies (Tables 17.5 and 17.6). There was an increase in the saturated hydrocarbons dominated by the 2 C₅ and 2 C₇ groups, and a decrease in the 3 C₃ fractions of the oenocytes in relation to age (Table 17.5). The saturated groups increased at the expense of the unsaturated groups, particularly in the case of 33:1.

Table 17.6 Changes in hydrocarbons in the wax gland oenocytes of the Cape honeybee, *A. m. capensis*, workers with age (Hepburn et al. 1991)

Hydrocarbon	Bee age (days)							
	0	3	6	9	12	15	18	21
25:0	23.8	23.0	21.9	26.9	26.6	30.0	29.9	31.0
27:0	38.8	37.2	35.8	36.1	40.7	44.0	47.8	51.7
29:0	4.5	6.4	7.5	12.0	9.7	8.0	6.3	2.0
31:0	3.0	2.6	2.8	2.8	3.5	2.0	3.0	3.4
33:0	6.0	6.4	4.7	4.6	5.3	4.0	4.5	3.4
35:0	1.5	1.3	0.9	0.9	1.8	2.0	3.0	1.7
Total	77.6	76.9	73.6	83.3	87.6	90.0	92.5	93.2
31:1	4.5	6.4	8.5	6.5	5.3	4.0	4.5	3.4
33:1	17.9	16.7	17.9	10.2	7.1	6.0	3.0	3.4
Total	22.4	23.1	26.4	16.7	12.4	10.0	7.5	6.8

The trends for the epidermal cells are similar, but on a smaller scale (Tables 17.5 and 17.6). Notable differences include an increase in the 2 C₉ pool, while 2 C₅ and 2 C₇ remained about the same.

Among the unsaturated groups, there was a marked reduction of 35:1 in the epidermis in relation to age (Table 17.5). The fatty acid profiles of the oenocytes and epidermal cells in relation to age are given in Tables 17.7 and 17.8 respectively.

While the total pool of saturated fatty acids in the oenocytes remained much the same, there were notable increases in 12:0, and decreases in 16:0 and 24:0, in relation to age (Table 17.7). Only minor changes occurred in the unsaturated fatty acids pool. The epidermal cells showed even fewer changes in fatty acid composition in relationship to age (Table 17.8).

Values for scale wax were based on samples harvested over several years in other age-related experiments. Thus, these values represented the already averaged content of thousands of individual wax scales taken from as many bees between the ages of 3- and 21- days-old (an internal control run established no differences between wax scale samples of that were freshly secreted or 2 years old.) Because of the necessity to pool wax scale samples, the data of Tables 17.5, 17.6 and 17.7 were re-expressed as total averages for direct comparison with scale wax in Tables 17.8 and 17.9. The former provided insight into the metabolic activities of the wax gland tissues, on an age-related basis, the latter allowed comparisons of average product content. The average content of scale wax hydrocarbons showed a 50 % reduction in the saturated 2 C₅ groups, compared with the two wax gland tissues (Tables 17.9 and 17.10). Also, the C₃₃ hydrocarbons of the oenocytes were far less than those of either the epidermis or scale wax. The other hydrocarbons were much the same for tissue and scale wax (Table 17.7). In the case of the fatty acids, there were large differences between the short chain (C₁₂ and C₁₄) and the long chain (C₂₄ to C₂₈) groups in both the tissues and the scale wax. There were also notable differences between the tissues and scale wax among the unsaturated fatty acids (Table 17.10).

Table 17.7 Changes in fatty acids in the wax gland oenocytes of the Cape honeybee, *A. m. capensis*, workers with age (Hepburn et al. 1991)

Fatty acid	Bee age (days)							
	0	3	6	9	12	15	18	21
12:0	0.0	0.0	0.0	0.0	3.1	7.0	14.1	28.8
14:0	4.4	4.6	4.8	3.9	4.2	3.5	2.5	2.5
16:0	19.3	17.6	15.0	14.7	14.5	12.0	10.9	8.4
18:0	4.1	3.9	4.1	4.4	4.7	4.8	5.2	4.4
20:0	2.2	1.9	1.7	1.9	1.8	1.9	1.8	1.7
22:0	1.2	1.1	1.2	1.6	1.8	2.0	2.2	1.9
24:0	48.5	48.9	50.8	50.9	54.3	49.2	45.2	34.8
26:0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
28:0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Total	79.7	77.0	77.6	77.4	79.4	80.4	81.9	82.5
16:1n9	2.3	3.7	4.7	4.6	3.9	3.2	3.0	2.8
18:1n9	4.5	5.4	5.3	5.5	6.0	4.9	3.6	3.9
20:1n9	1.5	1.9	1.4	1.5	1.1	1.0	1.0	0.9
Total	8.3	11.0	11.5	11.6	11.0	9.1	7.6	7.6
18:2n6	7.4	6.7	6.6	6.6	6.8	6.7	6.6	6.3
18:3n6	2.0	1.5	1.3	1.3	1.4	1.3	1.4	1.4
20:4n6	1.2	1.3	1.2	1.1	1.2	1.1	1.1	0.9
Total	10.6	9.5	9.1	9.0	9.4	9.1	9.1	8.6
18:3n3	1.0	1.3	1.6	1.7	1.1	1.0	1.2	1.0
20:5n3	0.4	0.4	0.4	0.5	0.4	0.3	0.4	0.4
Total	1.4	1.7	2.0	2.2	1.5	1.3	1.6	1.4

17.5 Secretion of Beeswax

The amounts of wax borne on average by worker bees of different ages are shown in Fig. 17.9. Paired comparisons of different age groups showed that not all age groups differed significantly. It is nonetheless worth commenting on the magnitude of the standard deviations. It requires between 24 and 48 h for any particular honeybee worker to produce a moderate-sized wax scale (Hepburn and Muller 1988). Moreover, on harvest, one cannot tell whether an individual honeybee, with no or only a little wax on it, is because it either did not secrete any wax, or had recently removed its scales and added them to the comb-building in progress. Consequently, one cannot relate any specific amount of wax back to a defined zero time. However, the general trend of the data is highly significant, and fully supported by the one-way analysis of variance. Thus, the amount of wax borne per bee is significantly affected by the age class of the bee.

When an adult worker bee emerges from its cell, the cuticle of the wax mirror is about 3 μm thick and, unlike other regions of the exoskeleton which increase in thickness with age (Menzel et al. 1969), it remains the same. Its basic ultra-structure has already been described (Locke 1961; Hepburn 1986; Hepburn et al.

Table 17.8 Changes in fatty acids of the wax gland epidermis of the Cape honeybee, *A. m. capensis*, workers with age (Hepburn et al. 1991)

Fatty acid	Bee age (days)							
	0	3	6	9	12	15	18	21
12:0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
14:0	2.5	2.5	2.9	3.1	2.8	2.9	2.8	2.7
16:0	26.5	23.8	25.9	24.7	26.2	25.6	25.9	25.3
18:0	10.0	7.0	5.5	4.6	3.3	3.9	3.7	7.6
20:0	0.3	0.3	0.2	0.3	0.3	0.4	0.3	0.2
22:0	0.6	0.6	0.6	0.6	0.4	0.3	0.4	0.2
24:0	21.5	25.8	24.4	27.2	25.0	20.4	23.5	22.5
26:0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
28:0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Total	61.4	59.9	59.6	60.4	58.1	53.5	56.7	58.5
16:1n9	2.4	2.4	2.1	2.2	2.3	2.1	1.9	2.0
18:1n9	8.0	7.7	7.7	8.4	9.5	9.0	8.5	8.1
20:1n9	2.4	2.4	2.6	2.5	2.6	2.9	2.7	2.6
Total	12.8	12.6	12.4	13.1	14.3	14.0	13.1	12.8
18:2n6	18.1	16.7	17.7	16.2	19.3	22.9	21.0	20.7
18:3n6	0.6	0.6	0.3	0.3	0.3	0.4	0.4	0.4
20:4n6	6.8	9.9	9.9	8.1	7.6	8.8	8.4	7.4
Total	25.4	27.2	27.8	26.6	27.2	32.1	29.8	28.5
18:3n3	0.3	0.3	0.2	0.4	0.4	0.4	0.5	0.3
20:5n3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Total	0.3	0.3	0.2	0.4	0.4	0.4	0.5	0.3

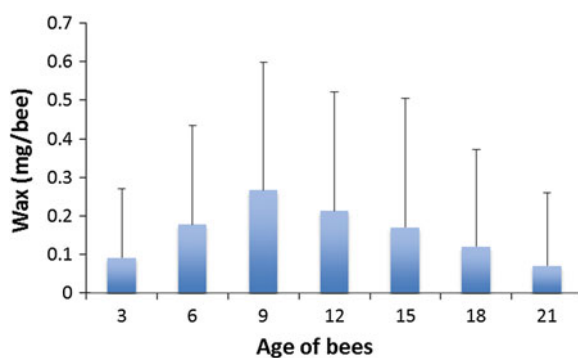
Table 17.9 Composition (percentage) of hydrocarbons in the wax gland tissues and waxes of the Cape honeybee, *A. m. capensis*, workers (Hepburn et al. 1991)

Hydrocarbon	Oenocytes	Epidermis	Scale wax	Comb wax
25:0	26.6	22.2	11.4	6.6
27:0	41.5	37.2	39.8	33.3
29:0	7.0	9.0	8.1	13.8
31:0	2.8	3.5	4.1	8.9
33:0	4.6	12.9	14.6	15.4
35:0	1.6	2.8	3.3	3.3
Total	84.3	87.6	81.3	81.3
31:1	5.4	–	–	–
33:1	10.3	4.2	4.9	6.5
35:1	0.0	8.2	13.8	12.2
Total	15.7	12.4	18.7	18.7

1991; Cassier and Lensky, 1995). The epidermis was earlier reported to lack both dermal glands and, more importantly, smooth endoplasmic reticulum (SER) during peak wax secretion (Sanford and Dietz 1976; Hepburn et al. 1991). This oversight was later amended by Cassier and Lensky (1995) who provided electron

Table 17.10 Average composition (percentage) of the fatty acids in wax gland tissues and waxes of the Cape honeybee, *A. m. capensis*, workers (Hepburn et al. 1991)

Fatty acids	Oenocytes	Epidermis	Scale wax	Comb wax
12:0	6.6	–	–	–
14:0	3.8	2.7	8.3	4.7
16:0	14.1	25.4	15.2	20.2
18:0	4.5	5.7	4.9	1.4
20:0	1.9	0.3	1.8	1.8
22:0	1.6	0.5	5.0	3.3
24:0	47.8	23.7	28.3	35.8
26:0	–	–	3.0	2.9
28:0	–	–	1.9	2.1
Total	80.3	58.4	68.4	72.1
16:1n9	3.5	2.8	0.9	1.9
18:1n9	4.9	8.4	18.2	15.0
20:1n9	1.3	2.6	–	–
Total	9.7	13.1	19.1	16.9
18:2n6	6.7	19.1	7.2	6.9
18:3n6	1.5	0.4	3.5	2.0
20:4n6	1.1	8.4	–	–
Total	9.3	28.1	10.7	8.9
18:3n3	1.2	0.4	1.8	2.1
20:5n3	0.4	–	–	–
Total	1.6	0.4	1.8	2.1

Fig. 17.9 Wax secretion (mg) by individual *A. m. capensis* worker honeybees of different ages (Hepburn et al. 1991)

micrographs of SER in epidermal cells. The idea that the epidermis has no role in the actual synthesis of beeswax is not new (Holz 1878). The major role of the epidermis in the production of wax appears to be the development of an elaborate system of small transport tubules (Reimann 1952; Locke 1961; Hepburn 1986; Cassier and Lensky 1995). The most notable and dynamic feature of the oenocytes is the abundant SER, whose rise and fall are synchronized with measured periods of secretion (Hepburn and Muller 1988), and which is considered to be indispensable for lipogenesis.

On the other hand, adipocytes are the primary site of intermediary metabolism in insects (Downer 1985; Keeley 1985), and the large quantities of lipid, protein and glycogen in the adipocytes associated with the wax gland support this generalization. The early mobilization of lipid from the adipocytes (Table 17.11) suggests that it might produce beeswax precursors. However, the absence of communicating junctions between adipocytes and oenocytes, and the fact that adipocyte lipid reserves are depleted prior to both the maximal development of the oenocyte SER and wax production, mitigates against this possibility. Likewise, at maximal wax production, the lipid content of the adipocyte is more or less constant. Finally, paraffins are synthesized by oenocytes and triglycerides by the adipocytes of locusts (Diehl 1973), and in beetles, lipid oxidation proceeds in oenocytes after they have taken up lipid droplets through the plasma membrane reduction–oxidation system of the adipocytes (Romer et al. 1974). Collectively these observations do not support an adipocyte origin for beeswax lipids.

Although the fine structure of the wax mirror cuticle and its wax transport tubules have now been visualized (cf. Hepburn 1986; Cassier and Lensky 1995), there remains the problem of the physical transport of beeswax precursors. Kurstjens et al. (1990) reported a partial characterisation of the proteins of wax scales and comb wax, in which some 17 fractions were separated. Two of these fractions have been implicated in wax precursor transport, on the grounds that their molecular weight distributions closely approximate those of known honeybee apolipoproteins. Thus, it is highly probable that hydrocarbons and fatty acid precursors of beeswax may be synthesized in the oenocytes, an interpretation strongly supported by the data of Hepburn et al. (1991), and then transported through the haemolymph to the surface of the insect in the form of primary or modified apolipoproteins (Kurstjens et al. 1990; Hepburn et al. 1991), probably derived from the epidermis (Cassier and Lensky 1995).

Before comparing the chemical content of the wax gland tissues with that of scale wax, it is important to note that the hydrocarbon and fatty acid contents of *A. m. capensis* comb wax (Tables 17.9 and 17.10) are virtually identical to those of its sister-race, *A. m. scutellata*, as reported by Tulloch (1980); results which lend confidence to the analyses presented here. The nature and origins of the large differences between scale and comb wax (Tables 17.9 and 17.10) are post-secretory phenomena, and have been dealt with in detail elsewhere (Kurstjens et al. 1985; Davidson and Hepburn 1986; Hepburn and Kurstjens 1988; and cf. Chaps. 13 and 14). The general trends in the hydrocarbon profiles of the oenocytes include an age-related increase in the saturated components (Table 17.6), reflecting considerable synthetic activity. By comparison, the epidermal hydrocarbons showed more modest changes in relation to the ages of the bees (Table 17.5); these are pronounced among the more minor groups, the unsaturated compounds. The hydrocarbons of the epidermis probably reflect oenocyte-derived material in transit because its age-related changes in hydrocarbons are not synchronized with the cycle of secretion.

There is an apparent discrepancy between the high C₂₅ and low C₃₅ content of the oenocytes vis-à-vis wax scales; but it could be that C₃₃ is formed from C₂₅ and

Table 17.11 Volume changes in the wax gland adipocytes of the Cape honeybee, *A. m. capensis*, workers with age (Heppburn et al. 1991)*

Feature	Bee age (days)										
	0	4	6	9	12	14	16	18	21		
Whole cell ^a	65 ± 26	110 ± 52	130 ± 100	117 ± 48	123 ± 52	120 ± 87	143 ± 65	95 ± 37	97 ± 32		
RER ^b	2	8	5	4	4	3	3	2	2		
Mitochondria ^b	5	10	8	8	10	9	11	12	13		
Lipid droplets ^b	61	24	22	21	17	25	18	17	21		
Protein granules ^b	-	10	19	22	7	1	6	-	-		
Glycogen granules ^b	5	3	14	6	10	5	7	10	7		

*Adipocyte volume ($\times 1000 \mu\text{m}^3$). Bees of age 0 days were significantly different ($P < 0.05$) from those of 4–16-day-old groups but not each other, but both were significantly different ($P < 0.05$) from the 4–16-day-old groups

^a whole cell volume

^b organelle volume

Table 17.12 Volume changes in the wax gland oenocytes of the Cape honeybee, *A. m. capensis*, workers with age (Hepburn et al. 1991)

Feature	Bee age (days)									
	0	4	6	9	12	14	16	18	21	
Whole cell ^a	8 ± 4	36 ± 13	54 ± 16	39 ± 10	45 ± 13	40 ± 14	52 ± 22	32 ± 10	30 ± 12	
SER ^b	65 ± 10	83 ± 14	82 ± 6	83 ± 24	81 ± 9	82 ± 10	8 ± 12	85 ± 12	74 ± 15	
Mitochondria ^b	10	9	8	8	8	9	7	5	7	
Glycogen granules ^b	23	7	8	9	9	7	6	7	15	

^a Oenocyte volume ($\times 1000 \mu\text{m}^3$). Bees of age 0, 4 and 6 days were significantly different ($P < 0.05$) from each other and from the groups 9–18-day-olds. But there were no significant differences within the 9–18-day-old group. Similarly, bees of 18- and 21-day-olds were not significantly different from each other but were different ($P < 0.05$) from the 9–18-day-old groups

^b Organelle volume density expressed as a percentage of mean oenocyte cytoplasm occupied by organelle. For SER, bees of age 0 and 21 days were significantly different ($P < 0.05$) from ages 4–18-day-olds. There were no significant differences between any of the 4–18-day-old groups

Table 17.13 Volume changes in the wax gland adipocytes of Cape worker, *A. m. capensis*, honeybees, with age (Heppburn et al. 1991)

Feature	Bee age (days)										
	0	4	6	9	12	14	16	18	21		
Whole cell ^a	65 ± 26	110 ± 52	130 ± 100	117 ± 48	123 ± 52	120 ± 87	143 ± 65	95 ± 37	97 ± 32		
RER ^b	2	8	5	4	4	3	3	2	2		
Mitochondria ^b	5	10	8	8	10	9	11	12	13		
Lipid droplets ^b	61	24	22	21	17	25	18	17	21		
Protein granules ^b	-	10	19	22	7	1	6	-	-		
Glycogen ^b granules	5	3	14	6	10	5	7	10	7		

^a Adipocyte vol (x 1000 μm^3). Bees of age 0 were significantly different ($P < 0.05$) from those of 4–6-day groups but there were no difference within the 4–6-day old groups. Similarly, bees of 18 and 21-days-old were not different from each other, but both were significantly different ($P < 0.05$) from the 4–6-day old groups

^a whole cell volume

^b organelle volume

C₂₇ outside the oenocytes. The fatty acid profiles of the epidermal cells lacked evident patterns of change consistent with the ageing of the bees or with the cycle of wax synthesis and secretion (Table 17.8)—excepting C₁₈. By contrast, large differences among the fatty acid pools of the oenocytes related both to the ages of the bees and to the cycle of synthesis (Table 17.7) and secretion (Fig. 17.6). Likewise, the average composition for fatty acids in oenocytes more closely mirrors those of scale wax than that of the epidermis (Table 17.8). The presence and increase of C₁₂ in the oenocytes coupled to its absence from epidermis and scale wax (Tables 17.7, 17.8 and 17.10), further suggests that the oenocytes perform chain elongation reactions. The decrease in C₁₆ and C₂₄ in the oenocytes over time is also consistent with synthesis and subsequent export. The oenocytes are the only cells of the wax gland complex whose developmental fate closely matches periods of wax synthesis (Tables 17.11, 17.12 and 17.13).

Unlike those of the epidermis, the hydrocarbon and fatty acid profiles of isolated oenocytes shared much in common with newly secreted wax scales. That the oenocytes are the probable source of beeswax hydrocarbons is supported by the close cyclical changes in ultrastructure that coincide with age-related cycles of secretion of beeswax by worker honeybees. These interpretations are consistent with the histochemical data of Reimann (1952), the autoradiographic studies of Piek (1964), studies of hydrocarbon synthesis in other insects by Diehl (1973, 1975), and with the electron microscopical study results of Cassier and Lensky (1995). Finally, it must be remembered that comb wax also mediates the acquisition of nestmate recognition cues in honeybees. Indeed comb wax in the colony, and the hydrocarbon layer of the epicuticle most probably serve as continuous media for hydrocarbon-soluble substances used by honeybees in nestmate recognition (Breed et al. 1988, et seq.) This aspect of the hydrocarbon story is further developed in Chap. 13.

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